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PROFILING OF BIOACTIVE COMPOUNDS IN WATER CRESS (*ENHYDRA FLUCTUANS* LOUR) BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract

Phytochemical test, HPLC analysis, antioxidant, and antibacterial activity were carried out with ethanol extract of *Enhydra fluctuans* Lour. Preliminary phytochemical screening revealed the presence of alkaloid, flavonoids, tannins, glycosides, steroids and saponin in the plant. In HPLC analysis, four ploy phenolic compounds e.g. (+)-catechin hydrate (CH), vanillic acid (VA), *p*-coumaric acid (PCA), ellagic acid (EA) were identified from crude extract, while five compounds e.g. CH, caffeic acid (CA), PCA, EA, KF (kaempferol) and two polyphenolic compounds e.g. gallic acid (GA) and KF were identified from aqueous ethanol and n-hexane fractions, respectively. It is the first time when bioactive polyphenolic compounds were detected in *E. fluctuans* extract by widely recognized HPLC method. The analysis of TLC plate revealed qualitative indication of the presence of bioactive antioxidant components in plant extract. Further studies are required to isolate this bioactive compounds and should be directed to carry out investigation for the biologically active components in order to determine their exact mechanism of action, and to improve nutritional profile and health benefits as well as to prepare natural pharmaceutical products of high value.

Keywords: *Bioactive compounds, Enhydra fluctuans Lour, TLC method, antioxidant, High Performance Liquid Chromatography*

Introduction

Bioactive compounds have a direct or indirect effect on living tissue, which provide benefits for health. Many bioactive substances with known effects on human physiology and disease have been identified through studies of plants used in traditional medicine, some of these plants are also food plants, or the same compounds also occur in food plants (1). The protection afforded by the consumption of plant products such as fruits, vegetables and legumes is mostly associated with the presence of phenolic compounds (2). Polyphenols are the most abundant antioxidants in the diet and are widespread constituents of vegetables, fruits, dry legumes, cereals, chocolate, and beverages (3, 4). Plant foods, which are rich sources of phenolic can act as antioxidants to reduce inflammation (5-7), prevent heart disease (8-10), lower the incidence of cancers (11-13) and diabetes (14) as well as reduce rates of mutagenesis in human cells (11, 15, 16).

Enhydra fluctuans Lour is an edible, semi aquatic, herbaceous vegetable plant, commonly known as '*Helencha*' belonging to family Asteraceae, distributed in tropical and subtropical regions (17). The plant is an edible, prostate, and spreading with slightly bitter serrate leaves, which are used to treat inflammation, skin diseases, laxative, bronchitis, nervous affection, leucoderma, biliousness and good in small pox (17, 18). This medicinal plant containing various phytochemicals such as flavonoids, alkaloids, saponins, tannins, phenols, β-carotene, protein and carbohydrates (18-23). Reportedly, many studies have suggested that consumption of this vegetable has many beneficial effects such as anticancer (24), antioxidant (23, 25, 26), antidiabetic (27), anti-inflammatory (28), antimicrobial (29, 30), hepatoprotective, anthelmintic and thrombolytic (23) and even neuropharmacological effects (17, 18, 31).

There has been a growing interest in the discovery and application of natural antioxidants in the food industry due to potential adverse effect of synthetic antioxidants (32). Globally a small proportion of plant species has been investigated both phytochemically and pharmacologically (33). The crucial factor for the ultimate success of an investigation into bioactive plant constituents is to have efficient systems available for the rapid chemical and biological screening of the plant extracts selected for investigation. Several methods e.g. paper chromatography (PC) and thin-layer chromatography (TLC) (34), high performance liquid chromatography (35), gas chromatography (GC) (36, 37), spectrophotometry (2, 34, 38) have been used for the identification, quantification of phenols and flavonoids from plant materials. In the last more than two decades, HPLC is gaining popularity among various analytical techniques in phytochemical and analytical chemistry to identify, quantify and purify the individual components of the mixture (39-41). Although numerous studies in the literature exist on *E. fluctuans* Lour derived constituents and their uses, no report has been found yet on detection of bioactive compounds of water cress (*E. fluctuans* Lour) by HPLC. Therefore, this study is to outline the diverse bioactive compounds in *E. fluctuans* Lour by HPLC method for the simultaneous separation, detection and quantification of phenolic compounds in a single run, which may act as antioxidants agent or other uses.

Methods

Sample collection

The fresh whole plants of *E. fluctuans* Lour were collected from local market, Khulna, Bangladesh. The collected plants were separated from undesirable materials, cleaned, washed with distilled water and sun dried for 17 days. Then the dried whole plants were ground into a coarse powder by a grinding machine (Capacitor start motor, Wuhu motor factory, China). The powder was stored in an airtight container and kept in a cool, dark and dry place until the analysis commenced.

Extraction

About 300g of powered material was extracted in 700 ml of 95% ethanol. The extract was underwent a coarse filtration by a vacuum filter with a piece of clean, white cotton material and Whatman filter paper twice. Then the filtrate was dried. Two solvent, n-hexan and aqueous ethanol were used for solvent-solvent partition. Both the n-hexan and aqueous ethanol fractions were evaporated under ceiling fan until dried. Weight of the n-hexan and aqueous ethanol fraction

comprised 59.4% and 40.6%, respectively of *E. fluctuans* Lour extract.

Phytochemical group test

With a view to detect the presence of various chemical constituents in *E. fluctuans* Lour, the crude ethanol extract was qualitatively analyzed for the presence of tannins, alkaloids, steroids, flavonoids, saponins and glycosides according to the standard procedure. Each of the tests was qualitatively expressed as negative (–) or positive (+) (Table 1). Distilled water, ethanol and n-hexane were used as solvent.

Determination of bioactive compounds in HPLC analysis

Polyphenolic composition in the *E. flutuans* ethanol extract was determined by HPLC analysis as per with some modifications (42). The analysis was carried out on a Thermo Scientific DionexUltiMate 3000 Rapid Separation LC (RSLC) systems (Thermo Fisher Scientific Inc., MA, USA), coupled to a quaternary rapid separation pump (LPG-3400RS), Ultimate 3000RS auto sampler (WPS-3000) and rapid separation diode array detector (DAD-3000RS). Separation was performed on Acclaim® C18 (4.6 × 250 mm; 5µm) column (Dionix, USA) at 30°C. The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C). For identifying and quantifying the polyphenols, the gradient method was applied as 5%A/95%B (0-9 min), 10%A/80%B/10%C (10-19min), 20%A/60%B/20%C (20-29 min) and 100%A (30 min) and 5 min post run with solvent A. For UV detection, the wavelength program was optimized as follows: 280 nm (0-18.0 min), changed to 320 nm (19-24 min) and finally to 380 nm (25-30 min) and the diode array detector was set at an acquisition range from 200 to 700 nm. Throughout the analysis, the flow rate was kept constant at 1 mL/min and the injection volume was 20 µL.

For the calibration curve, a standard stock solution (100 µg/mL) of each phenolic compound was prepared in methanol and final concentration was obtained at 1.0-5.0 µg/mL for gallic acid, (+)-catechin hydrate, vanillic acid, (-)-epicatechin, *p*-coumaric acid, ellagic acid, myricetin, kaempferol; 0.5-4.0 µg/mL for (+)-catechin hydrate, caffeic acid, rutin hydrate and 0.25-3.0 µg/mL for quercetin. The calibration curves were constructed from chromatograms as peak area vs. concentration of standard. Extract solution (5.0 mg/mL) was prepared in ethanol by vortex mixing (Branson, USA) for 30 min. Prior to HPLC analysis, all solutions were filtered through 0.20 µm nylon syringe filter (Sartorius, Germany) and then degassed in an ultrasonic bath (Hwashin, Korea) for 15 min.

Determination of antioxidant activity

The ethanol extract of *E. fluctuans* Lour in non-polar (n-hexane), medium polar (crude ethanol) and polar medium (aqueous ethanol) were used for the determination of antioxidant activity by TLC method. After applying 10% H_2SO_4 on the TLC plates, the TLC plates were viewed under UV detector (257 nm wavelength). A lot of colored and fluorescent positive spots under UV detector indicated the presence of antioxidant components in the sample (Fig.5 and Fig. 6). Components viewed in UV detector are marked by the sign ( ). Since n-hexane fraction exhibited better separation of compounds on TLC plate, 0.4g n–Hexane extract was used in column chromatography containing 20g silica-60 gel for further separation. After column separation, 37 sub fractions were obtained and TLC analysis was performed on each sub–fraction (Fig. 2).

Results

Phytochemical screening

Different phytochemical components of ethanol extract of *E. fluctuans* e.g. flavonoids, tannins, glycoside, and steroid were found to present in crude ethanol, n-hexane and aqueous fractions of *E. fluctuans* Lour, while alkaloid was found to present in both crude ethanol, n-hexane fractions and saponin was found to present only in crude ethanol fraction (Table 1).

HPLC analysis

The HPLC chromatogram of standard and ethanol extract of crude ethanol, n-hexane and aqueous ethanol fraction are shown in Fig. 1, 2, 3 and 4. In the experiment, seven polyphenolic compounds (GA, CH, EA, VA, PCA, CA and KF) were identified during HPLC analysis of three samples of *E. fluctuans* Lour (Table 2). Among them, three phenolic components of low content were detected in only one solvent e.g. 3.34 mg/100g of dry extract

VA in crude extract, 15.59 mg/100g of dry extract GA in n-hexane fraction, 4.97 mg/100g CA in aqueous ethanol fraction. KF was detected in both n-hexane fraction and aqueous ethanol fraction as 2.42 and 1.67 mg/100g of dry extract, respectively. The crude ethanol extract and aqueous ethanol fraction of *E. fluctuans* Lour contained a high concentration of EA as 251.24 and 234.27 mg/100g of dry weight, respectively, while CA and PCA were detected in fairly low content as 16.32 and 8.44, 0.97 and 0.90 mg/100g of dry weight, respectively.

Antioxidant activity

Since antioxidants are important to protect tissues from oxidative damage caused by reactive oxygen species, and nutrients contribute to remedy problems of malnourishment and obesity, and *E. fluctuans* Lour has traditional use as food because of preference of natural antioxidant in diet, the aquatic plants employed in this study was analyzed for antioxidant activities. In this experiment, the antioxidant level was measured *in vitro* by TLC method in crude extract, n-hexane fraction, sub-fractions of n-hexane and aqueous ethanol fraction of ethanol extract of *E. fluctuans* Lour (Fig. 5-6). A well-known antioxidant, ascorbic acid (Avocado Research Chemicals Ltd, Shore Road, Heysham, Lancs) was used as positive control. TLC plates run in all solvent systems exhibits spots on chromatogram indicating the presence of antioxidant components in the sample (Fig. 5 & Fig. 6). However, the TLC plates that was run in non-polar (n-hexane: Acetone = 3:1) and polar ($CHCl_3$: CH_3OH: H_2O =40:10:1) solvent systems exhibits more spots on chromatogram than the TLC plate run in medium polar ($CHCl_3$: CH_3OH =5:1) solvent (Fig. 5) indicating that compounds of *E. fluctuans* Lour were not separated well in medium polar solvent. On the other hand, spots were too congested in polar solvent system, while plant extract form comparatively separated spots in non-polar solvent system. Therefore, antioxidant compounds are separated well by using non-polar solvent and n-hexane fraction was used for further fractionation by column chromatography using silica-60 (Fig. 6). There were no spots at the lower region from sub-fraction 1 to 18. However, lower region spots become increased gradually from sub fraction 19 to 35 while upper region spots gradually disappear. This phenomena indicates that the compounds from later sub fractions are more polar than the compounds from earlier sub fractions.

Discussion

The vegetable plant, *E. fluctuans* is traditionally used to treat different diseases. In phytochemical screening of present experiment, different components of ethanol extract of *E. fluctuans* e.g. flavonoids, tannins, glycoside, steroid, alkaloid and saponin were found to present. The previous phytochemical screenings of *E. fluctuans* Lour also indicate the presence of alkaloids, glycosides, flavonoids, tannin and saponin (18-23). The biological activities of this plant may be due to the presence of these various groups of chemical compounds. Flavonoids are highly diversified plant pigments that possess antioxidant property and diverse biological activities e.g. anti-inflammatory, antiulcer, anti-viral, anti-cancer, anti-diabetic and cytotoxic (43-45). Many tannin molecules show anticarcinogenic and antimutagenic potentials related to their antioxidative property and antimicrobial properties (46). Saponins are steroid or triterpenoid glycosides are known to produce anti-inflammatory (47), cytotoxic (48), hypocholesterolaemic and anticarcinogenic activity (49). Plants containing saponins are major ingredients in traditional Chinese medicine (50). Steroids which are very important compounds due to their relationship with compounds such as sex hormone.

The present experiments also revealed that the ethanol extract of *E. fluctuans* Lour is a potential source of natural bioactive molecules, seven ploy phenolic compounds are identified in polar, medium polar and non-polar solvent by using HPLC. Phenolic compounds has a major contribution to antioxidant activity (8). The analytical method employed in this study is the first development of HPLC method for identification and analysis of bioactive compounds in *E. fluctuans* Lour. The ethanol extract of *E. fluctuans* Lour also possess antioxidant activity. Ethanol extract of *E. fluctuans* Lour exhibits higher antioxidant activity than chloroform and pet-ether extracts for the DPPH scavenging activity, NO-scavenging activity and super oxide scavenging activity (51). Further studies are required to isolate this bioactive compounds, to

determine their exact mechanism of action and to improve nutritional profile and health benefits as well as to prepare natural pharmaceutical products of high value.

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Table 1. Phytochemicals screening of *E. fluctuans* Lour

Phytochemical Group	Test performed	Standard	Result Crude ethanol extract	n-hexane fraction	Aqueous ethanol fraction
Alkaloid	Mayer's test	Nicotine	+	+	-
	Dragendroff's test		+	+	-
Flavonoids	General test	Rose petal/ Quercetin	+	+	+
Tannins	Ferric chloride (5%)	Catechu/ Gallic acid	+	+	+
	Potassium dichromate test		+	+	+
Glycoside	Molisch's test	Digoxin/ Tomato	+	+	+
Steroid	Salkowski's Test	Norgestrol	+	+	+
Saponin	Froth test	Detergent	+	-	-

(+) Indicates the presence of chemical constituents & (-) Indicates the absence of chemical constituents

Table 2. Used concentration, obtained average area and contents of polyphenolic compounds from mixed standards and three different samples (n=3) of *E. fluctuans* Lour

Polyphenolic compound	Mixed standards		Crude extract				n-Hexane fraction				Aqueous ethanol fraction			
	Conc. (µg/ml)	Avg. area	Con. (µg/ml)	Average area	Con. (mg/100g of dry extract)	% RSD	Con. (µg/ml)	Average area	Con. (mg/100g of dry extract)	% RSD	Con. (µg/ml)	Average area	Con. (mg/100g of dry extract)	% RSD
GA	5.0	4538.196	10000	-	-	-	10000	1429.238	15.59	0.73	10000	-	-	-
CH	4.0	1390.189		573.010	16.32	0.38		-	-	-		296.314	8.44	0.15
VA	5.0	11479.90		775.716	3.34	0.05		-	-	-		-	-	-
CA	4.0	4910.547		-	-	-		-	-	-		615.770	4.97	0.08
EC	5.0	2152.048		-	-	-		-	-	-		-	-	-
PCA	5.0	26059.00		515.122	0.97	0.01		-	-	-		474.792	0.90	0.02
RH	4.0	3033.851		-	-	-		-	-	-		-	-	-
EA	5.0	424.195		2243.664	251.24	5.37		-	-	-		2092.138	234.27	6.01
MC	5.0	11393.0		-	-	-		-	-	-		-	-	-
QU	3.0	6330.564		-	-	-		-	-	-		-	-	-
KF	5.0	11342.70		-	-	-		553.490	2.42	0.09		381.958	1.67	0.04

Conc. = Concentration; Con. = Content; GA = gallic acid; CH = (+)-catechin hydrate; VA = vanillic acid; CA = caffeic acid; EC = (-)-epicatechin; PCA= *p*-coumaric acid; RH = rutin hydrate; EA = ellagic acid; MC = myricetin; QU = quercetin; KF = kaempferol.

Figure 1. HPLC chromatogram of a standard mixture of polyphenolic compounds. Peaks: 1, gallic acid; 2, (+)-catechin; 3, vanillic acid; 4, caffeic acid; 5, (−)-epicatechin; 6, *p*-coumaric acid; 7, rutin hydrate; 8, ellagic acid; 9, myricetin; 10, quercetin; 11, kaempferol.

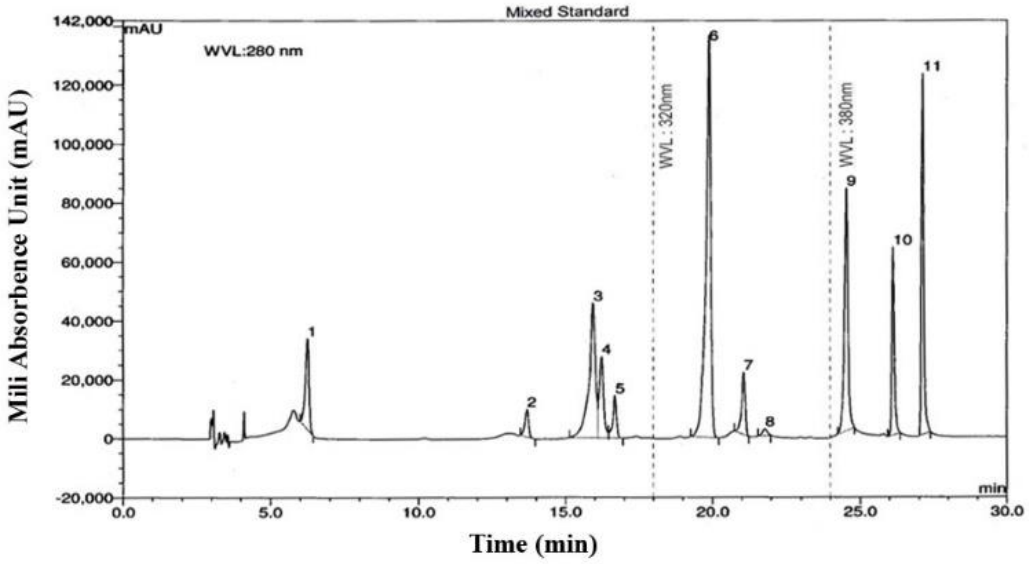

Figure 2. HPLC chromatogram of ethanol extract of crude ethanol fraction of *E.fluctuans* Lour. Peaks: 1, (+)-catechin; 2, vanillic acid; 3, *p*-coumaric acid; 4, ellagic acid.

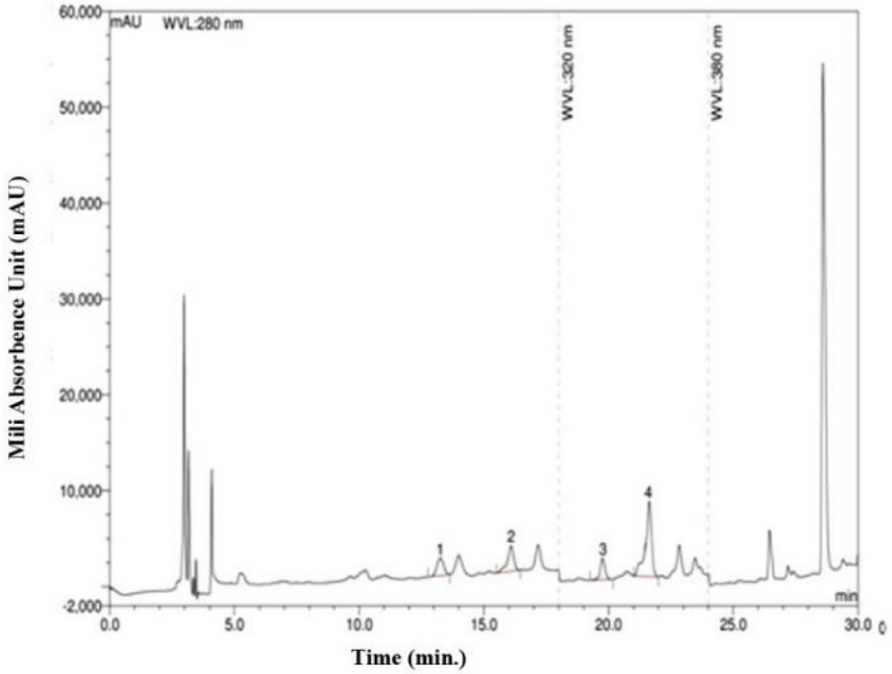

Figure 3. HPLC chromatogram of ethanol extract of n-hexane fraction of *E. fluctuans* Lour. Peaks: 1, gallic acid; 2, kaempferol.

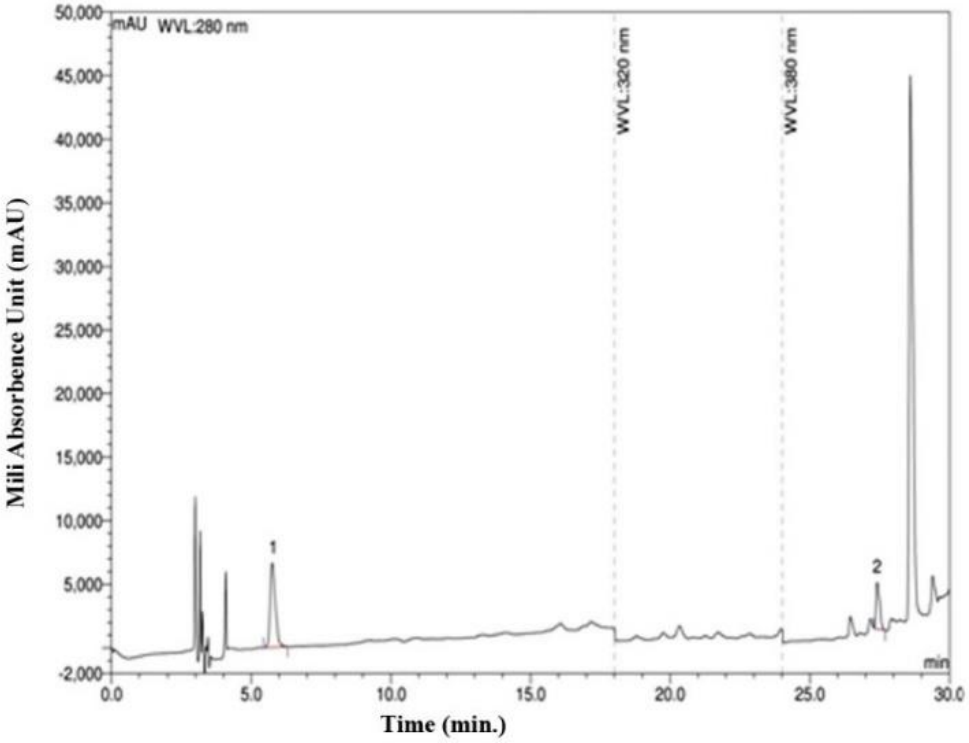

Figure 4. HPLC chromatogram of ethanol extract of aqueous ethanol fraction of *E. fluctuans* Lour. Peaks: 1, (+)-catechin; 2, caffeic acid; 3, *p*-coumaric acid; 4, ellagic acid; 5, kaempferol.

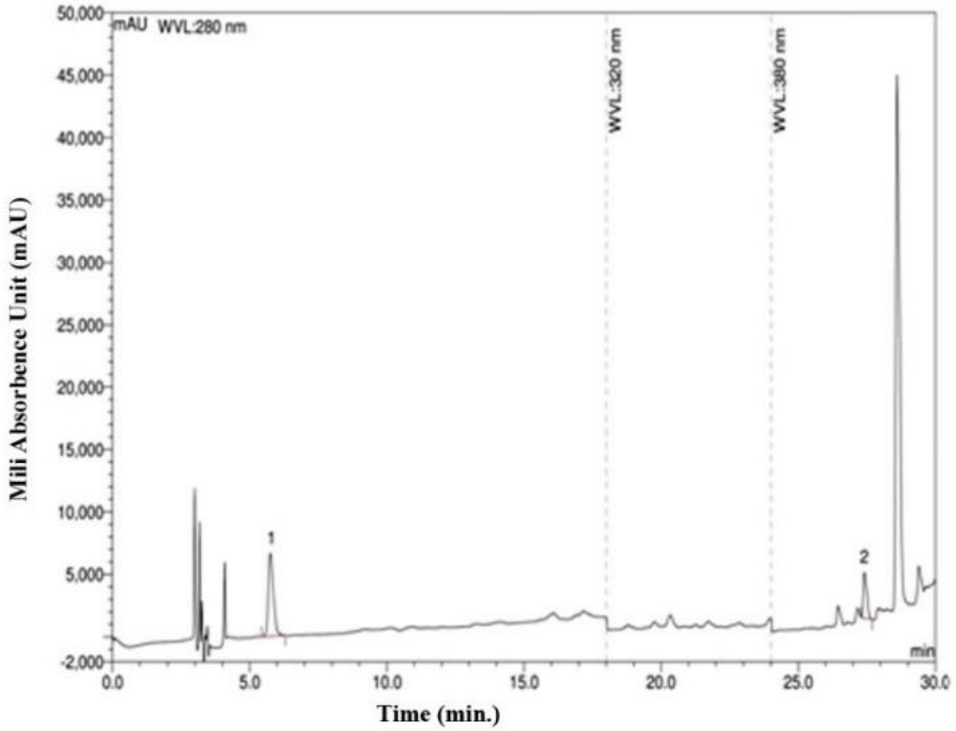

Figure 5. Antioxidant potential of crude ethanol extract of *E. fluctuans* Lour in non-polar, medium polar and polar medium. Aa: Ascorbic acid.

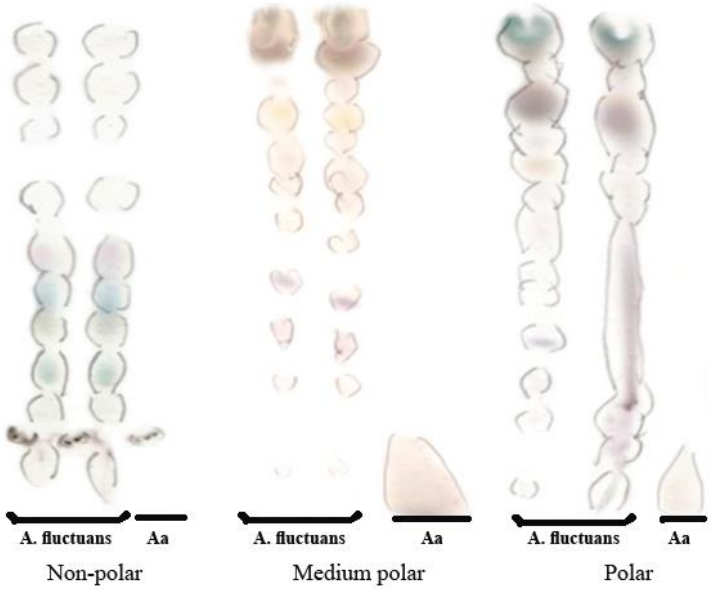

Figure 6. Comparison among TLC Plates of sub-fractions of n-hexane fraction of *E. fluctuans* Lour.